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Interaction and mixed aggregation of proteins from Tobacco mosaic virus strains

By Satyabrata Sarkar

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By SATYABRATA SARKAR

Aus dem Max-Planck-Institut für Biologie, Abt. MELCHERS, Tübingen, Corrensstraße 41

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Proteins from four strains of TMV, namely *vulgare*, *flavum*, *dahlemense* and *Holmes' rib grass*, were electrophoretically examined singly and in pairs at different hydrogen ion concentrations.

In weakly alkaline media an average of six polypeptide chains of TMV-protein remain associated together, while constant dissociation and reassociation takes place. This dynamic state of equilibrium is responsible for transient reciprocal associations of proteins or polypeptide chains from the first three TMV-strains. On lowering the p_H value, these interacting proteins form mixed aggregates with intermediate mobilities. Protein from the fourth strain, *Holmes' rib grass*, when tested against the strain, *vulgare*, neither showed any interaction in alkaline media nor formed mixed aggregates.

Factors determining the formation of mixed aggregates and the possible relationship among the four strains have been discussed.

The protein-part of a single Tobacco mosaic virus particle consists of 2130 ± 40 polypeptide chains which are very probably identical with one another [1] (FRANKLIN, CASPAR and KLUG, 1959). A polypeptide chain of the common TMV strain, *vulgare*, has a molecular weight of about 17 500 and consists of 157* amino acids (WITTMANN and BRAUNITZER, 1959)[2] whose sequence has been recently worked out[3] (ANDERER et al., 1960).

[1] R. E. FRANKLIN, D. L. D. CASPAR and A. KLUG, in: Plant Pathology, Problems and Progress 1908—1958, Univ. of Wisconsin, Pp. 447—461 [1959].

* According to a recent letter from Professor W. M. STANLEY addressed to Professor G. MELCHERS a polypeptide chain of TMV protein contains 158 amino acids and not 157 as found here earlier. This result could now be confirmed also by Dr. H. G. WITTMANN.

[2] H. G. WITTMANN and G. BRAUNITZER, Virology **9**, 726 [1959].

[3] A. ANDERER, H. UHLIG, E. WEBER and G. SCHRAMM, Nature [London] **186**, 922 [1960].

The polypeptide chains of several other TMV strains differ more or less in the composition and sequence of their amino acids [4, 5] but they all possess one remarkable common property: they can associate near their respective isoelectric points in an orderly way producing cylindrical protein-coats, typical of intact TMV-particles which were studied electrophoretically by KRAMER and WITTMANN, 1958 [6], as well as KLECZKOWSKI, 1959 [7]). The capacity is inherent in the structure of the polypeptide chains themselves since cylinders of variable length are produced even without the presence of a ribonucleic acid core.

At neutral and moderately alkaline p_H the TMV protein has often been reported to exist as small aggregates of six polypeptide chains with a molecular weight of about 100 000 and has been given the name "A-protein" as it was obtained by alkaline splitting of TMV. The size of an aggregate is, however, a function of such factors as p_H, ionic strength, temperature, protein concentration and so on. On high dilution, at high alkalinity or by the action of various organic solvents, the protein falls apart into single polypeptide chains which undergo easy denaturation (ANDERER, 1959 [8], ANSEVIN and LAUFFER, 1959 [9], WITTMANN, 1959 [10]).

On the basis of these observations, it seemed interesting to investigate:

1. Whether in a mixture of proteins from two different TMV strains an exchange of polypeptide chains occurs, and

2. whether mixed aggregates can be formed on acidification of such a mixture of proteins.

These investigations were undertaken with a view to finding out how far an exchange of polypeptide chains and formation of mixed aggregates are determined by the number of charged groups per polypeptide chain and by their structural similarities and differences.

Four strains of TMV, *vulgare*, *flavum*, *dahlemense* and *Holmes' rib grass*, were available, of which the amino acid compositions are now known (WITTMANN, 1960 [5]). *Flavum* and *vulgare* resemble each other very closely; each polypeptide chain of the former contains one aspartic acid less and one alanine more than the latter and most probably one amide group more than the latter on the basis of electrophoretic measurements (KRAMER and WITTMANN, 1958 [11]). As compared to both of them, *dahlemense* shows several quantitative differences and contains one methionine per polypeptide chain which is not present in the other two strains [5]. *Holmes' rib grass* strain differs very widely from all the three above both qualitatively and quantitatively. Each polypeptide chain of *Holmes' rib grass* contains several glutamic acid residues, three residues of methionine and one histidine, the last named being absent from the other three strains (KNIGHT, 1949 [4], WITTMANN, 1960 [5]).

Exchange of polypeptide chains and the formation of mixed aggregates were detected by the method of free electrophoresis since the proteins of the four strains have characteristically different mobilities (KRAMER and WITTMANN, 1958 [6]).

Present observations speak for mutual interaction and mixed aggregation between proteins from closely related strains of TMV.

I. Material and Methods

Isolation of Virus. After multiplication on greenhouse grown tobacco plants each strain of virus was isolated in a pure form by the conventional method of differential centrifugation with alternate freezing and thawing. A preliminary removal of associated plant proteins from expressed sap was achieved either by heat-denaturation at 60 °C for 10 minutes or by an isoelectric precipitation at p_H 4.3 (COMMONER et al., 1950 [12]; WITTMANN 1959 [13]). As the isoelectric point of *Holmes' rig grass* strain lies near about 4.5 (OSTER 1951 [14]; GINOZA and ATKINSON 1955 [15]), only a heat-denaturation treatment was adopted for the primary clearing of the virus containing plant-sap.

Splitting of virus and purification of A-protein. About 10 cm³ of a 1 – 3% virus suspension in dilute phosphate

[4] C. A. KNIGHT, J. biol. Chemistry **171**, 297 [1947].
[5] H. G. WITTMANN, Vergleichende Strukturuntersuchungen an Tabakmosaikvirus-Stämmen verschiedenen Verwandtschaftsgrades. Communication to the meeting of the Deutschen Botanischen Gesellschaft, Köln 1960 and Virology **12**, No. 4 [1960].
[6] E. KRAMER and H. G. WITTMANN, Z. Naturforschg. **13 b**, 30 [1958].
[7] A. KLECZKOWSKI, Virology **7**, 385 [1959].
[8] A. ANDERER, Z. Naturforschg. **14 b**, 24 [1959].
[9] A. T. ANSEVIN and M. A. LAUFFER, Nature [London] **183**, 1601 [1959].
[10] H. G. WITTMANN, Experientia [Basel] **15**, 174 [1959].
[11] E. KRAMER and H. G. WITTMANN, Communication No. 2-25, Proceedings of the 4th International Congress of Biochemistry, Vienna 1958.
[12] B. COMMONER, F. L. MERCER, PH. MERILL and A. J. ZIMMER, Arch. Biochem. Biophysics **27**, 271 [1950].
[13] H. G. WITTMANN, Z. Vererbungslehre **90**, 463 [1959].
[14] G. OSTER, J. biol. Chemistry **190**, 55 [1951].
[15] W. GINOZA and D. E. ATKINSON, Virology **1**, 253 [1955].

buffer ($m/50$) of p_H 7 was dialysed with stirring against one litre of glycine-NaCl-NaOH buffer of p_H 10.3 – 10.4 (p_H 10.6 in case of HR-strain) and an ionic strength of 0.1 at +4 °C for 12 to 16 hours. The unsplit or incompletely split TMV particles still present were centrifuged down in the cold in a Spinco preparative ultracentrifuge at about 60000 g for 60 minutes, and the clear supernatant was dialysed for 12 to 24 hours against glycine-NaCl-NaOH buffer of p_H 9.4 and ionic strength 0.04. The protein was purified by the method of countercurrent electrophoresis from the nucleic acid fraction at p_H 9.4 using the 10 cm^3 standard cell of a "Phywe" electrophoresis apparatus. A potential gradient of 9 – 10 Volt/cm for 8 to 10 hours was employed. As a criterion of purity, the ratio of absorption at 260 and 280 mμ was kept as low as possible. The values for the proteins of the four strains abbreviated as *AV*, *AF*, *AD* and *AHR* were 0.54; 0.56; 0.58 and 0.53 respectively indicating that the protein-preparations were practically free of nucleic acid (cf. KRAMER and WITTMANN 1958 [6]).

Concentration of protein was calculated from the extinction at 280 mμ, since a good agreement exists between A_{280} and nitrogen content. A sedimentation coefficient of $S_{20}\,W \approx 4$ at p_H 8 was measured for each type of A-protein.

Electrophoresis studies of A-protein mixtures at different p_H-values. Electrophoretic mobility measurements were done at 3.2 °C bath temperature in a "Phywe" electrophoresis apparatus. Errors in the magnification factor of the Schlieren camera and in the cross-sectorial area of Tiselius cell have been eliminated by using the same limb of the same cell for all comparative runs (ALBERTY and MARVIN 1950 [16]). To avoid small fluctuations in p_H or ionic strength a large volume of buffer solution was prepared for a whole series of experiments, kept at 4 °C and checked for p_H and conductivity from time to time.

AV, *AF* and *AD* solutions, each containing 0.4% protein and *AHR* containing 0.25% protein were dialysed separately in Michaelis buffer of p_H 8.0 and of an ionic strength, $I = 0.037$. This ionic strength, which is half of that used by KRAMER and WITTMANN (1958) [6] was chosen to achieve greater numerical differences among the mobility values of the A-proteins and consequent better separation of the Schlieren peaks. The combinations and concentrations of the protein mixtures are given in Table 1. Each mixture was studied electrophoretically within one hour from the moment of mixing of the respective A-protein pair.

After the runs the solution was recovered from the Tiselius cell and after standing for a week at 4 °C reexamined electrophoretically.

For aggregation experiments, the first three mixtures of table 1 were dialysed successively against Michaelis buffer of p_H 6.5, 5.5 and 5.0 ($I = 0.037$) for four hours each and then overnight against acetate buffer of p_H 4.92, $I = 0.037$, all in a cold room at +4 °C. The

Mixture type	p_H	Concentration of each protein in the final mixture [gm/100 ml]
1 $AV + AF$	8.0	0.2
2 $AV + AD$	8.0	0.2
3 $AF + AD$	8.0	0.2
4a $AV + AHR$	8.0	0.13
4b $AV + AHR$	8.5	0.12

Table 1. Protein mixtures used for electrophoretic studies.

mixture 4 a was acidified slowly by dialysis against buffers of gradually lower p_H values and examined electrophoretically at every stage. As *AHR* precipitated out easily at p_H 5.0 and also for reasons described later, the mixture 4 b was dialysed directly against a Michaelis buffer of p_H 5.2, $I = 0.037$.

The aggregated protein mixtures were split again into the constituent A-proteins by dialysis against glycine-NaCl-NaOH buffer of p_H 10.4 and their electrophoretic diagrams at p_H 8.0 and 8.5 were compared with their corresponding original mixtures.

II. Results

The ascending Schlieren pattern was sharper than the descending one but the deviation from enantiography was not too great when each protein was present alone. Anodic mobility values, expressed as $(\mu/\text{sec})/(\text{V/cm})$ are given in table 2. Each value is the average of several determinations with a potential gradient of 7.5 to 9.5 V/cm. The values were remarkably uniform with fluctuations mostly within ±2%.

The descending peak has generally a slightly lower mobility value, a phenomenon of common experience (ALBERTY, 1953 [17]).

The ascending boundary of *AHR* at p_H 8.0 has, however, a lower mobility than the descending one. An explanation for this discrepancy has to be sought in the nature of the *AHR* molecule itself, since all other factors are kept constant. However, the higher mobility-value of the descending boundary of *AHR* at p_H 8.0 might be due to dissociation of the imidazole ring of histidine. This amino acid is not present in the other three virus strains. It is also worth noting that the difference between the mobilities in the two arms disappeared by simply raising the p_H-value to 8.5.

[16] R. A. ALBERTY and H. H. MARVIN, J. phys. Colloid Chem. 54, 47 [1950].

[17] R. A. ALBERTY, in: The Proteins, vol. I A, edited by Neurath and Bailey, Academic Press 1953, P. 523.

Mobilities of the observed peaks with the mixtures 1, 2, 3 and 4 b (cf. table 1) are given in table 3 and the electrophoretic patterns are reproduced in fig. 1. In all mixtures, excepting *AV* + *AHR*, the mobilities of the observed Schlieren peaks are different from those of the pure components as presented in Table 2.

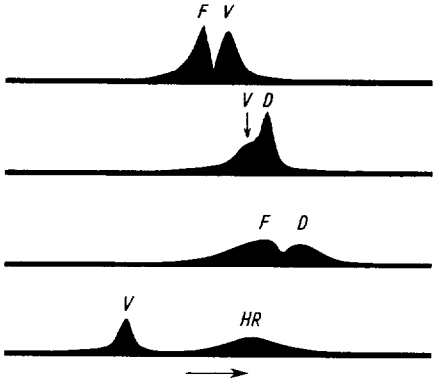

Fig. 1. Ascending-limb electrophoretic patterns of TMV-protein mixtures. *V*, *F*, *D* and *HR* stand for proteins from *vulgare*, *flavum*, *dahlemense* and *Holmes' rib grass* strains respectively. The first three diagrams are at p_H 8.0 while the *V* + *HR* mixture is at p_H 8.5.

Protein type	p_H	*U* ascending	*U* descending
AV	8.0	0.46	0.46
AV	8.5	0.47	0.47
AF	8.0	0.37	0.36
AD	8.0	0.59	0.57
AHR	8.0	0,67	0.71
AHR	8.5	0.84	0.84

Table 2. Anodic mobilities (U) of A-proteins in Michaelis buffer, $\Gamma = 0.037$, $U = (\mu/\text{sec})/(\text{V/cm})$.

The Schlieren peaks were always much better resolved at the ascending boundary. Due to incomplete separation and rapid spreading at the descending boundary, only one average mobility-value of this boundary has been given in some cases. From Table 3 it can be seen that: (1) mobility of a particular peak remains practically unchanged even after 7 days' standing of a protein mixture, (2) aggregation and resplitting does not affect the subsequent Schlieren pattern at p_H 8.0 and 8.5, (3) a fall in total protein concentration from 0.4% to about 0.1% after aggregation and resplitting hardly affects the mobility values and the Schlieren patterns. A comparison with table 2 indicates that (4) in all the three mixtures of *AV*, *AF* and *AD* the mobility of the observed peaks are not exactly the same as the corresponding proteins when each is present alone; the two peaks appear to approach each other so to say, while (5) the mobilities of *AV* and *AHR* are exactly reproduced in mixture. Different degrees of flattening of the diagrams are depicted, the sharpest being *AV* + *AHR* and the most overlapping and spreading is *AF* + *AD*.

On reducing the p_H value of the mixed solutions to 4.92 as described earlier, the proteins were found to aggregate as was evident from the opalescence of the samples. In table 4 are given the anodic mobilities ($U = (\mu/\text{sec})/(\text{V/cm})$) of aggregated proteins both alone and in mixture at p_H 4.92. Aggregated *dahlemense* protein moved as a single boundary while *vulgare* as well as *flavum* each formed two distinct peaks. According to KRAMER und WITTMANN (1958) [6] the faster peak, designated here as the main gradient, corresponds to rod-like aggre-

Mixture type	Total protein conc. [gm/100 ml]	p_H	Time after mixing	*U* ascending two peaks	*U* descending
AF + *AV*	0.40	8.0	1 hour	0.40 + 0.44	0.37 + 0.41
AF + *AV*	0.40	8.0	7 days	0.40 + 0.45	0.38 + 0.42
AF + *AV*	0.11	8.0	12 days *	0.39 + 0.44	0.43
AV + *AD*	0.40	8.0	1 hour	0.51 + 0.56	0.53
AV + *AD*	0.40	8.0	7 days	0.52 + 0.55	0.50 + 0.53
AV + *AD*	0.19	8.0	10 days *	0.52 + 0.56	0.50 + 0.53
AF + *AD*	0.40	8.0	1 hour	0.44 to 0.50 + 0.58+	0.33:0.46 + 0.49+
AF + *AD*	0.40	8.0	8 days	0.41 to 0.49 + 0.56+	0.34 + 0.47
AF + *AD*	0.18	8.0	10 days *	0.41 to 0.48 + 0.57+	0.35:0.46 + 0.51
AV + *AHR*	0.25	8.5	1 hour	0.47 + 0.85	0.45 + 0.83
AV + *AHR*	0.25	8.5	7 days	0.47 + 0.84	0.46 + 0.84

Table 3. Anodic mobilities of peaks observed in A-protein mixtures $(\mu/\text{sec})/(\text{V/cm})$. * After aggregation and resplitting. + The slower peak is flattened and broader than the faster peak.

gates of dimensions comparable with intact TMV-particles, while the slower (= second) gradient corresponds to discs and small incompletely aggregated units.

The failure of better reproducibility as seen in table 4 may well be traced back to the high p_H-mobility dependence at this p_H range (KRAMER and WITTMANN, 1958 [6]).

Sample No.	Composition	U = (μ/sec)/(V/cm) at p_H 4.92			
		ascending		descending	
		main peak	second peak	main peak	second peak
1	*AF*	0.77	0.68	0.64	0.65
2	*AF*	0.73	0.66	0.69	—
3	*AV*	0.99	0.91	0.99	0.97
4	*AV*	0.95	0.84	0.92	—
5	*AD*	1.09	—	1.08	—
6	*AD*	1.09	—	1.10	—
7	*AF* + *AV*	0.91	0.82	0.94	0.88
8	*AV* + *AD*	0.97	—	0.96	—
9	*AF* + *AD*	0.91	—	0.89	—

Table 4. Anodic mobilities of aggregates of A-protein and their mixtures.

Results indicate that in all the three cases mixed aggregates are produced. The formation of one single gradient from *AF* and *AD* with a mobility value almost exactly in the middle of those of the corresponding pure strains show that the two types of A-proteins possess sufficient similarity to be able to form mixed aggregates. The greater spreading of this gradient as compared to *AV* + *AD* (Fig. 2 a and 2 b) is a natural consequence of the fact that the difference in mobilities between *AD* and *AF* is greater than that between *AV* and *AD*. Therefore, even if the degree of heterogenity in composition remains the same, the mobility-value of the mixed aggregates will be scattered over a wider range in case of *AF* + *AD*.

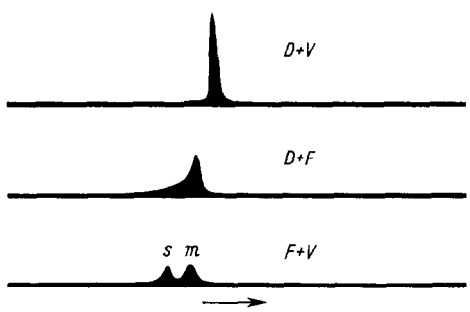

Fig. 2. Ascending-limb electrophoretic patterns of TMV-protein mixtures at p_H 4.9. m = main peak, s = second peak, other abbreviations as in Fig. 1. For explanation see text.

In case of *AV* + *AF*, although two separate peaks are seen (fig. 2 c), it is found that the main peak corresponding to protein cylinders has a mobility intermediate between the main *AV* and the main *AF* aggregates while the second peak corresponding to discs lies also intermediate between the corresponding second peaks of the pure aggregates (Fig. 3).

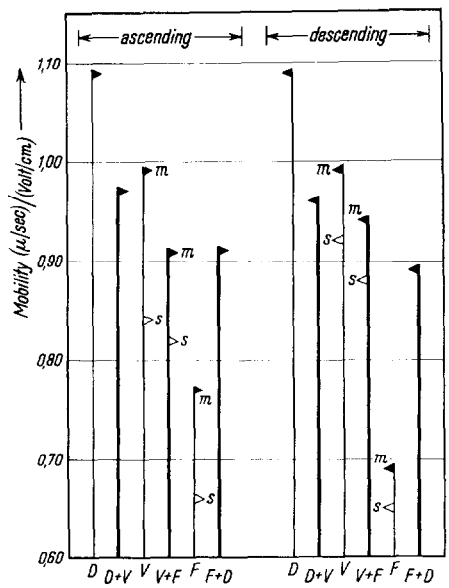

Fig. 3. Diagrammatic representation of the relative positions of schlieren peaks produced by TMV-proteins both alone and in binary mixtures at p_H 4.9, after an arbitrary but equal time of electrophoresis under identical conditions. Abbreviations as in figs. 1 and 2. For explanation see text.

It is, however, interesting to note that whenever *AD* is present, the united aggregates move as a single boundary. The tendency of *AD*-units to unite to long cylinders seems to be so strong that the free existence of *AV* and *AF* discs (cf. KRAMER and WITTMANN, 1958 [6]) is rendered difficult. In any case, the absence of peaks corresponding to pure *AF* and pure *AD* aggregates and very probably also of pure *AV* aggregates in the mixed solutions at p_H 4.9 speak strongly for the existence of mixed aggregates.

The turbidity observed in each mixed solution at p_H 4.9 was as far as possible removed by centrifuging at about 7000 *g* for 30 minutes in the cold and the opalescent supernatant was examined electrophoretically. The peaks observed maintained the same contour and mobility as before. After the run,

each solution was dialysed against glycine-NaOH buffer, p_H 10.4 to split the mixed aggregates again into their constituent A-proteins as described before. Electrophoresis in Michaelis buffer p_H 8.0 exhibited again the presence of two peaks of approximately equal concentrations as judged by their area; the mobilities have already been given in Table 3.

In sharp contrast to the formation of mixed aggregates among *vulgare*, *flavum* and *dahlemense* proteins, those of *vulgare* and *Holmes' rib grass* strain maintained their identities even in intimate mixture. As the p_H value of a mixture of *AV* and *AHR* was lowered stepwise (sample 4 a in Tab. 1), the *AHR* units aggregated into rods early. At p_H 5.5 there are at first three gradients with mobilities 0.39, 0.75 and 1.10, which correspond to *vulgare* A-protein, aggregated *HR* protein and aggregated *vulgare* protein respectively (Table 5). After about 14 hours of standing, the slowest peak corresponding to free *AV* practically disappeared, the fastest assumed a larger area showing the formation of more *vulgare*-aggregates, while the middle one (*HR*-aggregate) remained unchanged (Fig. 4). On further lowering the p_H value to 5.0 the solution became too turbid for electrophoretic runs.

p_H	$U = (\mu/sec)/(V/cm)$	
	AV	*AHR*
8.0	0.46	0.85
6.5	0.46	0.86
6.1	0.45	0.85
5.5	0.39 and 1.10*	0.75*
5.5	0.40 and 1.14*	0.79*
5.0	too turbid	

Table 5. Anodic mobilities (U) of observed peaks in a mixture of *AV* and *AHR* in Michaelis buffer of different p_H values. $\Gamma = 0.037$. (Results of sample 4 a from table 1.)
* Aggregated.

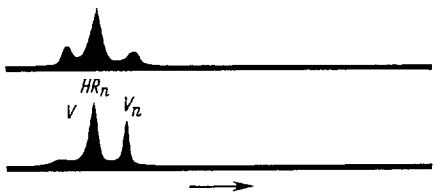

Fig. 4. Ascending-limb electrophoretic patterns at p_H 5.5 showing the gradual transformation of *vulgare* protein (*V*) into *vulgare*-aggregates (*Vn*) of higher mobility in the presence of aggregated *Holmes' rib grass* protein (*HRn*). The lower pattern was observed 14 hours after the first run (upper diagram). For explanation see text.

Obviously, no mixed aggregates were formed. During such a slow and stepwise lowering of the p_H, the aggregation of *HR*-protein is complete before *vulgare*-protein approaches its isoelectric point. To bring about quick aggregation, therefore, a mixture of *AV* and *AHR* at p_H 8.5 (sample 4 b of table 1) was dialysed directly against Michaelis buffer of p_H 5.2 of ionic strength 0.037. On analysis, two sharp peaks were obtained in both arms of the Tiselius cell with mobilities corresponding to pure *vulgare*- and pure *HR*-aggregates (Fig. 5 and Table 6), the deviation being well within the range of experimental errors. Both the gradients are symmetrical and the Schlieren pattern returns to the base line between peaks indicating the absence of mixed gradients having intermediate mobilities.

Fig. 5. Ascending-limb electrophoretic pattern of a mixture of *vulgare*- and *Holmes' rib grass* proteins at p_H 5.2.

Sample	$U = (\mu/sec)/(V/cm)$	
	ascending	descending
AHR alone	0.67	0.65
AV alone	1.09	1.06
AV + *AHR*	0.67 and 1.08	0.68 and 1.05

Table 6. Anodic mobilities of *AV* and *AHR* at p_H 5.2.

III. Discussion

The protein mixtures between *vulgare*, *flavum* and *dahlemense* in weakly alkaline media undergo varying degrees of interaction. Among the various types of protein-protein interactions, a simple dissociation and association of peptide chains has been reported from time to time, whereby either relatively stable complexes are formed or the different forms remain in a state of dynamic equilibrium. If the six polypeptide chains of *AV* and *AF* could dissociate freely and combine reciprocally with one another, the different mixed units would possess characteristic mobilities as listed below (Table 7), considering that the preferred size of the aggregates remains at six-polypeptide chains and that in such small aggregates all the charged groups of each type of polypeptide chain are electrokinetically active.

Configuration	Anodic mobility U = (μ/sec)/(V/cm)
a) $6\,AV$	0.460
b) $5\,AV + 1\,AF$	0.445
c) $4\,AV + 2\,AF$	0.430
d) $3\,AV + 3\,AF$	0.415
e) $2\,AV + 4\,AF$	0.400
f) $1\,AV + 5\,AF$	0.385
g) $6\,AF$	0.370

Table 7. Calculated mobilities of various mixed aggregates of *vulgare* and *flavum* proteins at p_H 8.0.

There are only two observed peaks with mobilities which correspond to b and f. It is difficult to visualise why only these two configurations should accumulate preferentially. The mobility values of $AV + AD$ and $AF + AD$ mixtures do not conform well to such 5 : 1 combinations and the electrophoretic diagrams speak more for a dynamic equilibrium.

Continuously interacting protein mixtures cause boundary anomalies in sedimentation and electrophoretic studies, which have been summarised and classified on the basis of the velocity constants of the forward and reverse reactions by ALBERTY and MARVIN (1950)[16], LONGSWORTH (1959)[18] and BROWN and TIMASHEFF (1959)[19] among others. FIELD and OGSTON (1955)[20] using human hemoglobin showed that the spreading of a boundary will be greater as the difference of velocities between the forms and their mean lives are greater. These boundary anomalies should not, however, be confused with the more common types of anomalies encountered with high protein concentrations in comparatively low ionic strengths of the buffer as pointed out by various workers (SVENSSON, 1944[21]), JOHNSTON and OGSTON, 1946[22], LONGSWORTH, 1947[23], and ALBERTY, 1948[24]). The non-existence of such factors under the conditions of the present investigations is corroborated by the absence of boundary anomalies with $AV + AHR$ mixture and by the constancy of mobilities over relatively wide ranges of protein concentrations.

An understanding of the dynamic state of equilibrium between the protein mixtures under consideration demands a knowledge of the mode and rate of dissociation of the proteins. This is difficult, since – as already stated in introduction – the size of an aggregate depends upon various interacting factors. Ultracentrifugal studies on dissociation of TMV-protein into single polypeptide chains have not provided definite clue as to whether the dissociation proceeds through definite steps or not (cf. ANSEVIN and LAUFFER, 1959[9], WITTMANN, 1959[10]). Without, therefore, attempting to ascribe definite mobility values for particular mixed aggregates in weakly alkaline media by postulating particular dissociation types as $6\,P \rightleftarrows 3\,P + 3\,P$, $6\,P \rightleftarrows 4\,P + 2\,P$ or $6\,P \rightleftarrows 5\,P + 1\,P$ where P stands for a single polypeptide chain, it can be stated that wherever mixed aggregates of transient existence are formed, it is evidenced by a flattening of the electrophoretic diagrams and by an approaching together of the respective Schlieren peaks. A simple case would be the one where a *vulgare* "A-protein"-unit associates with one of flavum, thus forming shortlived "double-A-protein-units" of intermediate mobility.

The marked deviation from enantiography in the two arms of the Tiselius cell is characteristic of reaction-boundaries. The descending boundaries could not be used to calculate exact mobilities for evident reasons but the ascending values were well reproducible. According to SVENSSON (1946)[25] "the generalisation that all disturbances are smaller on the descending side is no doubt erroneous and the omission of data from the ascending side involves an unjustifiable waste of experimental material". In contrast to the mobility-values of $AV + AF$ mixture at p_H 8.0 where both schlieren peaks appear to approach each other, it is remarkable that with $AD + AF$ mixture the mobility of the faster peak corresponds very closely to that of pure AD while there is no peak corresponding to pure AF on the ascending side. The difference in mobility between AD and AF is rather large and, therefore, on the ascending side, where the particles move out of the solution into the buffer above, some of the faster units will have a chance to escape the zone of rapid dissociation and reciprocal association. Once out of

[18] L. G. LONGSWORTH, in: Electrophoresis, edited by Milan Bier, Academic Press 1959, Pp. 91–136.
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the zone, the faster particles can gain their own mobility and thereby the front end of the schlieren diagram can move with a mobility equal to that of the pure faster component. The situation in the descending arm would be the reverse. On this side there is actually a small but clearly isolated peak corresponding to pure *AF*-units with a mobility 0.33 to 0.35 (Fig. 6).

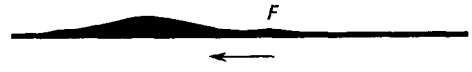

Fig. 6. Descending-limb electrophoretic pattern of a mixture of *flavum*- and *dahlemense*-proteins at p_H 8.0. Arrow indicates the direction of migration.

The proteins of *vulgare* and *Holmes' rib grass* strains do not obviously exchange their polypeptide chains with each other, though each of them is subject to reversible dissoziation. Both in weakly alkaline media and at $p_H \approx 5$ the observed peaks maintain the characteristic mobilities of pure *vulgare*- and pure *Holmes' rib grass*-proteins.

An explanation for the formation of mixed aggregates on lowering the p_H value of binary mixtures of *AV*, *AF* and *AD* described before is superfluous on the basis of the interactions observed in weakly alkaline media. Obviously, with an increase of the H-ion concentration, large aggregates are formed in which polypeptide chains of different strains are packed together in various possible combinations. On simple statistical grounds, particles of greater heterogenity will be more frequent than relatively homogeneous aggregates and thereby the observed peak will depict an intermediate mobility. Somewhat similar observations with hemocyanins from three different species of snails were reported by TISELIUS and HORSFALL (1939) [26]. Asymmetric recombination of different types of human and canine hemoglobins, on rapid neutralisation of a mixture of acid-split half-molecules has been reported by ROBINSON and ITANO (1960) [27].

Although mixed aggregation between proteins from *vulgare*, *flavum* and *dahlemense* strains of TMV have been observed, no such association was found between proteins of *vulgare* and *Holmes' rib grass* strains. The polypeptide chains of the latter, therefore, do not fit with those of the wild strain, *vulgare*. The conditions necessary for an orderly association are similarity or reciprocity in spatial configuration of the polypeptide chains as well as the number and mode of distribution of charged groups on them. The secondary and tertiary structures are certainly dependent on the primary structure, but hardly anything is known as to the maximum permissible variations in the composition and sequence of aminoacids in different mutants in order to just allow a mixed aggregation. In spite of clear-cut differences in the aminoacid composition [5] and total number of charged groups per polypeptide chain, the three TMV strains, *vulgare*, *flavum* and *dahlemense*, possess sufficient structural similarity for mixed aggregation. In weakly alkaline media *AV* possesses two negative charges more than *AF*, while *AD* has still two more negative charges. However, the difference in total charge between *AHR* and *AV* is even greater than that between *AF* and *AD*. This alone might prevent mixed aggregation but, as already mentioned, analytical results have demonstrated also radical qualitative and quantitative differences in amino acid composition of the *Holmes' rib grass* strain as compared to the other three. At the moment, therefore, it is not possible to differentiate between the roles played by major structural differences and differences in number and distribution of charged groups in the process of mixed aggregation between the TMV-proteins. Even then, it is interesting that in reconstitution-experiments the *Holmes' rib grass* protein can be successfully used with *vulgare*-nucleic acid (FRAENKEL-CONRAT and SINGER, 1959 [28]).

From the results presented above, two questions easily crop up; *viz.* 1) do such mixed proteins originate in a living host cell, and 2) what sort of "phylogenetic" relationships can be assumed between the four strains of TMV used here? If unambiguous experimental proof for simultaneous multiplication of two strains of a virus in one and the same host cell could be available, a treatment of the first question might enter the scope of experimentation. As to the second question, the three strains *vulgare*,

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flavum and *dahlemense* are certainly "related" to one another, particularly because *flavum* is a mutant of *vulgare*. *Holmes' rib grass* strain seems to stand quite apart. At the present moment any statement regarding possible relations in the history of origin of the *Holmes' rib grass* and *vulgare* would involve only speculations.

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